# Human cytomegalovirus *UL97* open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir

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**HUMAN cytomegalovirus (HCMV, a betaherpes virus) is the cause of serious disease in immunologically compromised individuals, including those with acquired immunodeficiency syndome[1]. One of the compounds used in the chemotherapy of HCMV infections is the nucleoside analogue 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (ganciclovir). The mechanism of action of this drug is dependent on the formation of the nucleoside triphosphate, which is a strong inhibitor of the viral DNA polymerase[2-4]. Thymidine kinase, which is encoded by many of the herpesviruses, catalyses the initial phosphorylation of ganciclovir. But there is no evidence for the coding of this enzyme by HCMV[2,5,6], and DNA sequence analysis of the HCMV genome has shown that there is no open reading frame characteristic of a herpesvirus thymidine kinase[7]. Here we present biochemical and immunological evidence that the HCMV *UL97* open reading frame codes for a protein capable of phosphorylating ganciclovir. This protein seems to be responsible for the selectivity of ganciclovir and will be useful tool in the understanding and refinement of the antiviral activity of new selective anti-HCMV compounds.**

In an attempt to identify the enzyme responsible for phosphorylation of ganciclovir in HCMV-infected cells, we observed that the amino-acid sequence coded by the HCMV *UL97* open reading frame (ORF) had some regions homologous to those of protein kinases. Furthermore, these sequences had homology with other proteins more distantly related to the protein kinases, such as the bacterial kinases that act on the aminoglycoside antibiotics[8]. To characterize HCMV UL97 and investigate its ability to phosphorylate ganciclovir, we expressed the protein in a heterologous prokaryotic system. The N-terminal regions of protein kinases are non-catalytic but they do have a role in the regulation of enzymatic activity[9-10]. We therefore cloned a truncated part of the HCMV UL97 ORF (positions 141,462-142,712 in the HCMV genome[7]) which did not code for the first 326 amino acids but retained all the sequences that align with the catalytic domains of the protein kinases. This truncated gene was cloned into the prokaryotic expression vector pGMT7 (G. Micklem, personal communication), which contains a bacteriophage T7 x10-s10 promoter/ribosome binding site controlling the expression of the inserted sequence[11]. This plasmid pGMT7-UL97tr directs the expression of a largely insoluble protein of $M_r$ 39,000 (39K), which is the predicted relative molecular mass of the open reading frame coded by the cloned DNA fragment (Fig. 1, lane 2). This protein, UL97tr, is not expressed in *Escherichia coli* BL21 cells containing the plasmid without the truncated UL97-coding insert (Fig. 1, lane 1). To confirm the viral specificity of the recombinant protein, purified UL97tr from BL21 was prepared by SDS-polyacrylamide gel electrophoresis and used to produce specific antisera. These sera reacted by western blotting with UL97tr and with a protein from HCMV-infected cells of $M_r$ 80K, which is the predicted value for UL97 (Fig. 1, lane 4). Some smaller weakly reacting proteins (lane 3) were found in infected cells; these may represent processed forms of UL97 or degradation products.

Having demonstrated the viral specificity of UL97tr, we tested its ability to phosphorylate ganciclovir by assaying extracts from BL21/pGMT7-UL97 that expressed UL97tr and control extracts prepared from BL21/pGMT7 but without the truncated UL97 insert (Fig. 2). Extracts from BL21/pGMT7-UL97 expressing UL97tr efficiently phosphorylated ganciclovir whereas control extracts did not. The low solubility of the UL97tr protein does not prevent the detection of enzymatic activity. Similar results have been found for other recombinant proteins[12] and it may be that some soluble active protein is present during the assay.

To show that the activity in these extracts was specific to recombinant UL97tr, we did several immunological experiments. Human sera from transplant patients having a high (post-transplant) or low (pre-transplant) titre to HCMV antigens (D. Morris, personal communication) were reacted with extracts from BL21 cells expressing UL97tr and the extracts assayed for residual enzyme activity (Fig. 3). The ganciclovir-phosphorylating activity in extracts of BL21/pGMT7-UL97 expressing UL97tr was neutralized by the HCMV immune serum but not by the non-immune serum. Furthermore, extracts from HCMV-infected cells were assayed for their ability to phosphorylate ganciclovir either with or without prior incubation with the UL97tr-specific antiserum. The antiserum against UL97tr neutralized the kinase activity of the extracts prepared from HCMV-infected cells (Fig. 3). To verify the specificity of the kinase activity of UL97 for ganciclovir, extracts from HCMV-infected or mock-infected cells were immunoprecipitated with antiserum against recombinant UL97tr and the preciptates assayed for kinase activity. The results in Fig. 4 shows that this activity immunoprecipitated with the UL97tr serum only from HCMV-infected cells and not from mock-infected cells. This observation is strong evidence that UL97 has an intrinsic kinase activity towards ganciclovir.

We have shown that extracts from both HCMV-infected cells and *E. coli* expressing recombinant UL97tr are able to phosphorylate ganciclovir, and have provided immunological evidence that suggests that the activity in both systems is specific to the protein product of the UL97 ORF. These results and those

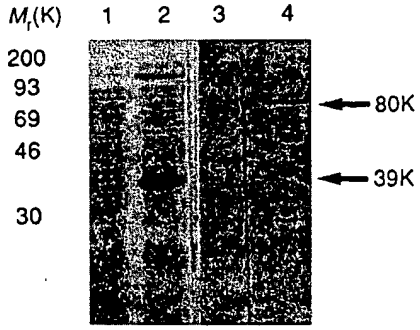


FIG. 1 Western blot analysis of UL97tr and of HCMV-infected cell extracts with UL97tr-specific antiserum. Lanes 1 and 2, proteins isolated from extracts of BL21 containing plasmid pGMT7 or plasmid expressing UL97tr, respectively. Extracts were prepared by sonification of BL21 cells in 50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA, 1 mM PMSF, 5 mM $\beta$-mercaptoethanol and 5 mg ml$^{-1}$ lysozyme, and after disruption of the cells in SDS buffer, proteins were run on 10% SDS-polyacrylamide gels. For lanes 3 and 4, proteins were prepared from MRC5 cells that had been either mock-infected or infected with HCMV strain AD169, respectively, for four days. After transfer to nitrocellulose, blots were probed using hyperimmune rabbit sera raised against purified recominant UL97tr protein as described[17]. Molecular weight calibration is shown on the left and was derived from standard markers stained with Coomassie brilliant blue. The 39K recombinant protein and the 80K protein are indicated.

in the accompanying letter[13], taken in conjunction with ganciclovir-resistance studies[14], show that UL97 is responsible for the phosphorylation of ganciclovir in HCMV-infected cells. The finding that an HCMV-coded enzyme related in sequence to protein kinases can phosphorylate a nucleoside analogue is unexpected. UL97 is also conserved in the other herpesviruses[8,15] and although the similarity between UL97 and the eukaryotic protein kinases is low[8], some of the other herpesvirus genes are more obviously related to the eukaryotic protein kinases. All of the herpesvirus sequences differ from the cellular enzymes at positions that are highly conserved in protein kinases. UL97 and its homologue in human herpesvirus-6 also differ significantly from the other herpesvirus sequences in regions that may be important for enzymatic activity, as determined from the crystal structure of cyclic AMP-dependent protein kinase[16]. One explanation for the activity seen here is that the UL97tr is modifying a bacterial protein in order to enable it to phosphorylate ganciclovir. We believe that this explanation is unlikely for the following reasons. In HCMV-infected human cells resistance to ganciclovir that is associated with reduced ganciclovir phosphorylation is due to a mutation in UL97 (ref. 13), and we have shown here that antiserum raised against UL97tr can efficiently prevent extracts from HCMV-infected human cells from phosphorylating ganciclovir. In addition, recombinant baculovirus expressing UL97 also phosphorylates ganciclovir (C. Olmstead, personal communication). These observations would demand that UL97 has the unlikely ability to modify a protein from human, insect and bacterial cells in such a way as to phosphorylate ganciclovir. It will be necessary to purify the UL97 before its ability to phosphorylate ganciclovir directly can be proven.

The role of UL97 in the replication of HCMV is not known, but unlike the herpes simplex virus homologue UL13 (D. McGeoch, personal communication), we have not yet found any protein kinase activity associated with UL97tr. One explanation may be that in the absence of a true HCMV-encoded thymidine kinase, the viral UL97 has evolved to substitute for this function. Alternatively, perhaps UL97 does function as a protein kinase, as predicted by amino-acid sequence comparison, and its ability to phosphorylate ganciclovir is a result of a chance similarity between ganciclovir and the natural substrate of the enzyme.

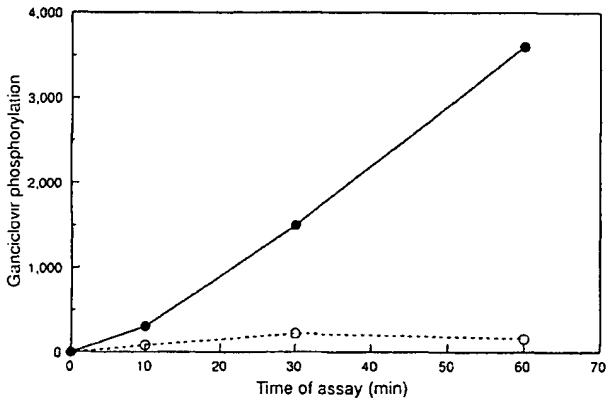


FIG. 2 Phosphorylation of ganciclovir by extracts of BL21 expressing the recombinant HCMV protein UL97tr (●), or by control extracts (○) without the UL97tr insert. Extracts of *E. coli* expressing UL97tr were prepared as described in Fig. 1 legend. Enzyme activity was assayed in 10 mM potassium phosphate, pH 8, 2 mM ATP, 2 mM dithiothreitol, 1 M NaCl, 1% nuclease-free BSA (Sigma) and 10 μM ganciclovir (containing 15 μCi [8-$^3$H]ganciclovir), and the extent of phosphorylation was determined as described[18]. Results are expressed as pmol ganciclovir phosphorylated per min by 1 mg of bacterial extract.

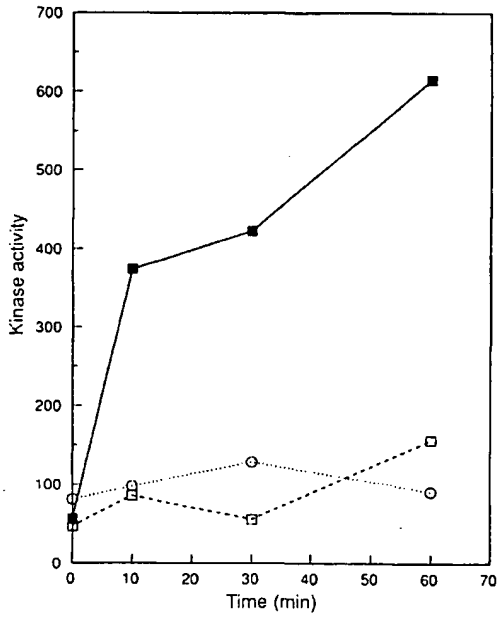


FIG. 4 Immunoprecipitation of ganciclovir kinase activity using UL97tr serum. Extracts from HCMV-infected MRC-5 cells or from mock-infected (○) cells were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% NP40 and 0.5% sodium deoxycholate) and mixed with rabbit serum reacting with non-immune serum (□) or with rabbit anti-UL97tr serum (■), diluted 1:20 in PBS. The resultant immune complexes were precipitated on staph A-Sepharose beads (Pharmacia) and the pellet was washed three times with RIPA buffer. Pellets were then resuspended in enzyme assay buffer and the ganciclovir kinase activity determined.

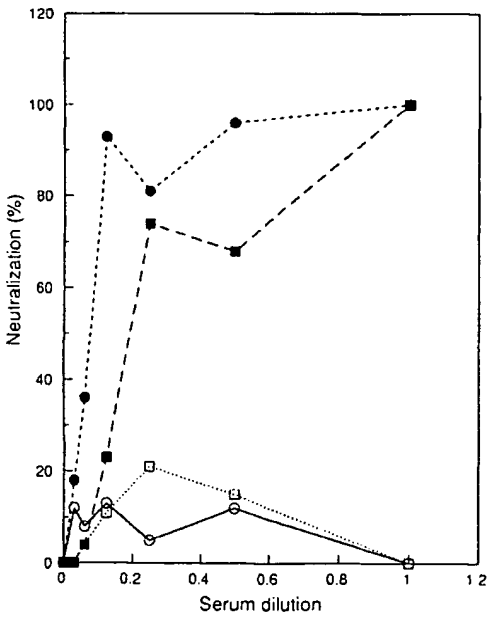


FIG. 3 Neutralization of ganciclovir kinase by antisera. Extracts prepared either from HCMV-infected MRC-5 cells or from BL21 cells containing plasmid pGMT7-UL97 were incubated with human HCMV immune (■) or non-immune serum (□), or with rabbit anti-UL97tr serum (●) or the respective preimmune control serum (○), at the dilutions indicated for 1 h at 4 °C, and then assayed for residual ganciclovir kinase activity. Results show percentage neutralization of ganciclovir kinase activity compared to enzyme to which no serum had been added.

Our results confirm that UL97 is an unusual member of the protein kinase sequence family. Characterization of the HCMV protein responsible for phosphorylating ganciclovir will help in the design of improved antiviral nucleoside analogues for treating HCMV infection. □

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# A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells

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**HUMAN cytomegalovirus (HCMV) is a major pathogen in immunosuppressed individuals, including patients with acquired immune deficiency syndrome. The nucleoside analogue ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl)-guanine) is one of the few drugs available to treat HCMV infections, but resistant virus is a growing problem in the clinic[1] and there is a critical need for new drugs. The study of ganciclovir-resistant mutants has indicated that the selective action of ganciclovir depends largely on virus-controlled phosphorylation in HCMV-infected cells[2–5]. The enzyme(s) responsible have not been identified. Here we report that the HCMV gene *UL97*, whose predicted product shares regions of homology with protein kinases, guanylyl cyclase and bacterial phosphotransferases[6–8], controls phosphorylation of ganciclovir in HCMV-infected cells. A four-amino-acid deletion of UL97 in a conserved region, which in cyclic AMP-dependent protein kinase participates in substrate recognition[9], causes impaired ganciclovir phosphorylation. The implications of these results for antiviral drug development and drug resistance are discussed.**

To map the HCMV-encoded function that controls ganciclovir phosphorylation, the viral genome of mutant 759$^r$D100, which is deficient in drug anabolism[4], was cloned into an overlapping set of nine cosmids, which were then used in marker transfer experiments (Table 1). Two non-overlapping cosmids, pC7S95 and pC7S37 (Fig. 1), transferred ganciclovir resistance to the wild-type parental strain AD169. The resistance marker of pC7S37 has been mapped to the viral DNA polymerase and

TABLE 1 Marker transfer of ganciclovir resistance

| Transfected DNA | Plating efficiency (%) in ganciclovir |
|---|---|
| — | <0.04 |
| pC7S2 | <0.03 |
| pC7S6 | <0.09 |
| pC7S11 | <0.17 |
| pC7S31 | <0.04 |
| pC7S37 | **2.3** |
| pC7S40 | <0.05 |
| pC7S47 | <0.23 |
| pC7S95 | **2.8** |
| pC7Sdl | <0.23 |

Marker transfer of ganciclovir resistance. Human foreskin fibroblasts were transfected with infectious wild-type AD169 DNA alone (—), or cotransfected with AD169 DNA and the indicated cosmid. Progeny virus from the transfected cells were tested for their ability to form plaques in 35 μM ganciclovir (per cent plating efficiency). Marker transfer of ganciclovir resistance by pC7S37 and pC7S95 is shown in bold. The preparation and maintenance of fibroblasts, preparation of infectious AD169 DNA, and details of marker transfer experiments will be described elsewhere (V.S. *et al.*, manuscript submitted). 759$^r$D100 viral DNA was prepared essentially as described for AD169, except MRC-5 cells were used and only extracellular virus was collected. After partial digestion with *Sau*3A, fragments of 20–30 kb were ligated into the *Bam*HI site of cosmid vector pC7108 (ref. 25), packaged into phage (BRL λ packaging system), and cosmid clones isolated by phage infection of *E. coli* (strain N4956) and selected with ampicillin. Cosmids pC7S2, 6, 11, 31, 37, 40, 47, 95 and dl were isolated, characterized by digestion with restriction enzymes and Southern blot analysis (results not shown), and partially digested with *Hin*dIII before use in marker transfer experiments.

shown to be unrelated to ganciclovir phosphorylation (V.S. *et al.*, manuscript submitted). In contrast, the ganciclovir-resistant recombinant virus GDG$^r$K17, isolated from marker transfer experiments with pC7S95, gave a value for the 50% effective dose of ganciclovir which was 3–10-fold higher than that of wild-type AD169 (data not shown) and was unable to induce the phosphorylation of $^{14}$C-labelled ganciclovir in infected cells (Fig. 2*a*). Similarly, GDG$^r$XBAF4, a ganciclovir-resistant recombinant virus isolated from marker transfer experiments with the F fragment of 759$^r$D100, produced by digestion with the restriction endonuclease *Xba*I (data not shown), was also unable to phosphorylate the drug (Fig. 2*a*). Thus, both a ganciclovir-resistance marker of 759$^r$D100, and the mutation responsible for the drug-phosphorylation defect are contained in the 13-kilobase (kb) overlap of the F fragment and pC7S95 (Fig. 1). Further marker transfer experiments with plasmids containing cloned 759$^r$D100 DNA fragments within the 13-kb overlap localized the ganciclovir-resistant marker to a 2.6-kb region of DNA containing the complete *UL97* and parts of the *UL96* and *UL98* open reading frames (Fig. 1). Ganciclovir anabolism studies of the ganciclovir-resistance recombinant viruses, GDG$^r$SAL4, GDG$^r$HS1 and GDG$^r$EH9, isolated from marker transfer experiments with plasmids pSAL7, pHS7 and pGEH7, respectively (Fig. 2*b*), confirmed that the mutation affecting ganciclovir phosphorylation was also contained in the 2.6-kb fragment. DNA sequence analysis of this region revealed only a single change when compared with the wild-type AD169 sequence: a 12-base-pair (bp) deletion within the *UL97* open reading frame (Fig. 3). These results show that the gene product of *UL97* controls ganciclovir phosphorylation in HCMV-infected cells and that mutations in this open reading frame can confer resistance to this drug.

Homologues of *UL97* are encoded by herpes simplex virus[10], varicella zoster virus[11], Epstein-Barr virus[12] and human herpesvirus 6 (HHV-6), which suggests that there may be a conservation of function among the α, β and γ human herpesviruses[6]. These genes encode regions of homology conserved among protein kinases and phosphotransferases[6,13,14]. The 12-bp deletion in